Advances in Brief

Protease Pretreatment Increases the Efficacy of Adenovirus-mediated Gene Therapy for the Treatment of an Experimental Glioblastoma Model[1]

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Abstract

Effective virus-mediated gene therapy for cancer will be facilitated by procedures that enhance the low level of gene transfer mediated by replication-deficient, recombinant viral vectors. We found recently that protease pretreatment of solid tumors is a useful strategy for enhancing virus-mediated gene transduction *in vivo*. In this study, we examined the potential of protease pretreatment to improve the efficacy of a gene therapy strategy for prodrug activation that depends on infection with a recombinant adenovirus encoding herpes simplex virus thymidine kinase (Ad-HSV-tk). Trypsin or a dissolved mixture of collagenase/dispase was inoculated into xenografts derived from the human glioblastoma multiforme-derived cell lines, U87 or U251. Ad-HSV-tk was administered 24 h after protease pretreatment, and animals were then treated for 10 days with ganciclovir (GCV). We found that protease pretreatment increased the efficacy of adenovirus mediated HSV-tk/GCV gene therapy in these experimental tumor models. Mice receiving Ad-HSV-tk/GCV after protease pretreatment demonstrated a significantly greater regression of tumors compared with those treated with Ad-HSV-tk/GCV alone. No adverse effects of protease pretreatment were observed. No signs of metastasis were seen either by histological inspection of lymph nodes or by a PCR-based analysis of selected mouse tissues to detect human tumor cells. Our findings indicate that protease pretreatment may be a useful strategy to enhance the efficacy of virus-mediated cancer gene therapy.

Introduction

GBM[3] is highly invasive and resistant to conventional radiotherapy and chemotherapy (1). Despite attempts at aggressive multimodality treatment regimens using surgery, radiation, and chemotherapy, there has been little progress in improving the outcome of patients with such tumors in the past decade. The current treatment of these brain tumors remains largely palliative (1). New therapeutic approaches, such as gene therapy, hold promise for the development of improved treatment strategies.

Therapeutic strategies that use gene transfer are being explored for the treatment of brain tumors (2, 3). Several gene therapy strategies appear promising based upon studies *in vivo* in animal models (4–9). Such approaches involve the transfer of genes encoding many different types of molecules including prodrug-activating enzymes, cytokines, and growth inhibitors. In virtually all cases, the efficacy of such therapies has been limited by low levels of transgene transduction. A

major obstacle to the successful application of gene therapy strategies that rely on *in vivo* virus-mediated transduction of tumor cells is the poor distribution of recombinant viral vectors throughout the tumor mass. Advances in gene therapy will depend on the development of vector delivery systems capable of the efficient introduction of genes into target cells.

Adenoviral vectors have been evaluated extensively for use in gene therapy because of their ability to be produced at high titers and to transfer foreign genes into a wide variety of nondividing and dividing cells (10–14). However, the use of these recombinant viruses is limited by poor transduction efficiency and a limited distribution of transgene delivery. This has been particularly problematic for the treatment of solid tumors in general and of brain tumors in particular (15–17). To address this problem and to enhance current gene therapy approaches for the treatment of GBM, we evaluated protease pretreatment as a means of enhancing viral vector-mediated gene transfer (18). In that study, we found that protease pretreatment of GBM-derived xenografts resulted in increased levels of viral transgene expression as a result of enhanced recombinant virus infection. To extend these observations, we have evaluated the usefulness of this strategy to improve the efficacy of a suicide gene therapy strategy dependent on the extent of gene transfer by Ad-HSV-tk for the treatment of animals bearing GBM-derived xenografts (19, 20). To characterize further the therapeutic potential of this strategy, the effects of the protease pretreatment on tumor invasion and metastatic tumor spread were also evaluated.

Materials and Methods

Cell Culture. The human GBM cell lines U87 and U251 were obtained from American Type Culture Collection. Cells were grown in DMEM and supplemented with 10% fetal bovine serum and penicillin/streptomycin in a humidified atmosphere of 5% CO_2.

Adenovirus Preparation. The recombinant replication-deficient adenovirus, Ad-βgal (kindly provided by Dr. Perry Nisson, University of Texas, Austin, TX), containing the *Escherichia coli* βgal gene under control of the Rous sarcoma virus long terminal repeat promoter, and Ad-HSV-tk encoding tk from HSV (provided by Dr. Arbans K Sandhu, Institute for Human Gene Therapy, Philadelphia, PA) were propagated in 293 cells and purified by CsCl density centrifugation. Recombinant adenovirus was titered by determination of the $TCID_{50}$ (tissue culture infectious dose for 50% of the cells). Viral stocks were stored in 10% glycerol and kept at −80°C until use.

Protease Treatment and Virus Infection of Immunosuppressed Mice Bearing GBM Xenografts. Female BALB/c homozygous nude (*nu/nu*) mice, 6 weeks of age, were maintained in a pathogen-free environment throughout the experiment. Animals were inoculated s.c. with cells from the human GBM-derived cell lines, U87 (7×10^6 cells) or U251 (5×10^6 cells). At 9 days after inoculation of the cells, animals with xenografts that were 0.6–0.7 cm in diameter were treated with intratumoral injections of PBS, PBS containing trypsin (100 μg; Sigma Chemical Co., St. Louis, MO), or PBS containing a mixture of collagenase and dispase (10 μg; Boehringer Mannheim, Inc., Indianapolis, IN). Each treatment consisted of 100 μl of saline or protease solution per tumor injected as 25 μl into each of the four tumor quadrants. Twenty-four h after protease treatment, Ad-HSV-tk or Ad-β gal was inocu-

laced directly into the center of the tumor (1×10^9 pfu in 50 μl of saline). After administration of Ad-HSV-tk or Ad-βgal, animals received an i.p. injection of GCV (50 mg/kg), a prodrug that requires activation by HSV-TK, twice daily for 10 consecutive days.

Tumor size was measured using calipers every 2 days, and tumor volume was determined using the simplified formula for a rotational ellipsoid: length (mm) $\times$ the square of the width (mm)$^2 \times \pi/6$ (Ref. 21). The tumor size of U87 and U251 flank xenografts was determined for 50 days after cell inoculation, and xenografts were excised and weighed at day 50. We examined daily an identically treated cadre of animals for survival until day 200 and scored them as dead when tumors increased to 20 mm in any dimension.

Examination of Secondary Metastasis in Mice Bearing GBM Xenografts. Metastases in mice-bearing GBM xenografts were evaluated in three ways: H&E staining of lymph nodes, fluorescence microscope examination of major organs in mice bearing xenografts of U251 cells transfected with a recombinant DNA construct encoding GFP; and PCR analysis of major tissues. To evaluate metastases to lymph nodes, all visible lymph nodes were dissected from the abdomen of mice bearing U87 xenografts on day 50 after cell inoculation. We evaluated mice from each of three groups: mock treated; treated with Ad-HSV-tk/GCV alone, and trypsin pretreatment combined with Ad-HSV-tk/GCV. Lymph node specimens were fixed in 10% formalin, embedded in paraffin, sectioned at 10-μm thickness, and stained with H&E.

To further examine the possibility of metastases of human tumor cells from the xenografts to major organs, xenografts were established in mice with U251 cells that had previously been stably transfected with an expression construct encoding GFP. Fifty days after the administration of Ad-HSV-tk/GCV therapy with trypsin pretreatment, tumor xenografts and major organs (lung, liver, and brain) were dissected, frozen in Tissue-Tek OCT compound (Sakura Finetek U.S.A., Torrance, CA), and stored until use at $-80°$C. Histological sections of these tissues were examined for the expression of GFP by fluorescence microscopy. Other mice bearing these xenografts were sacrificed on day 50 after the inoculation of tumor cells and evaluated for micrometastasis by PCR analysis of DNA from their major organs. We performed PCR analysis using primers for GFP to determine whether GFP-transfected tumor cells had metastasized to these tissues. PCR amplification was used to identify either a 484-bp GFP fragment or a 571-bp fragment from the β-actin gene using two pairs of primers: upper GFP primer, 5'-ACCCTGGTGACCACCCTGAC-CTAC-3' and lower GFP primer, 5'-GGACCATGTGATCGCGCTTCTCGT-3'; upper β-actin primer, 5'-ATGGATGACGATATCGCTCG-3' and lower β-actin primer, 5'-ATGAGGTAGTCTGTCAGGT-3'. The primer pair for mouse β-actin was used as a control for the PCR reaction. The conditions for PCR amplification were 30 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, followed by 72°C for 10 min. Reaction products were separated by gel electrophoresis on a 1.5% agarose gel and stained with ethidium bromide.

Evaluation of the Effect of Protease Administration on Normal Rat and Human Brain Tissue Architecture. Adult female Fischer 344 rats were anesthetized with an i.p. injection of 300 μl of 0.85% saline containing 60 mg/kg of ketamine and 7.5 mg/kg of xylazine. After the rats were immobilized in a stereotaxic apparatus, a linear incision was made over the bregma, and a burr hole was drilled in the skull 1 mm anterior and 3 mm lateral to the bregma bilaterally. Ten μl of saline or trypsin (100 μg) were injected at the depth of 3.5 mm below the dura, using a 25-μl Hamilton syringe with a 22-gauge needle. Twenty-four h later, the rats were euthanized, and the brains were removed, fixed in 10% formalin, and stained with H&E.

We obtained normal human brain, white and gray matter, for histological evaluation immediately after autopsy. Saline or 100 μg of trypsin mixed with black ink (10 μl) were injected directly into these tissues using a 25-μl Hamilton syringe with a 22-gauge needle. After 4 h incubation at 37°C in a humidified atmosphere of 5% CO_2, we fixed the specimens in 10% formalin, stained with H&E, and examined them histologically for morphological evidence of changes in tissue architecture.

Statistical Analyses. All statistical analysis was performed using Statview software (Abacus Concept, Inc., Berkeley, CA; 1994). Unpaired t test analysis was used to compare tumors receiving different treatments. Kaplan-Meier survival curves were calculated. Differences in survival between treatment groups were tested using the log-rank test.

Results

Effect of Protease Pretreatment on Xenograft Growth. Prior to the evaluation of protease as an adjunct to gene therapy, we sought to determine whether protease pretreatment itself affected the growth of GBM-derived tumor xenografts. We inoculated animals bearing tumor xenografts with intratumoral proteases prior to mock treatment with Ad-βgal (Fig. 1). Tumor xenografts in animals treated with either trypsin, collagenase/dispase, or PBS showed similar growth kinetics, and all tumors grew to the same final volume, ~3500 mm^3, with similar kinetics (Fig. 1). These results demonstrate that intratumoral protease inoculation has no effect on the growth of U87 GBM-derived tumor xenografts.

Effect of Protease Pretreatment on the Efficacy of Ad-HSV-tk/GCV Gene Therapy. To determine whether the enhanced gene transfer that we reported previously following protease treatment would improve the efficacy of gene therapy (18), we treated human GBM xenografts in mice with Ad-HSV-tk/GCV gene therapy. In preliminary experiments, we identified a dose of virus that was subtherapeutic and did not result in a complete inhibition of tumor growth (data not shown). Nine days after tumor cell inoculation, tumor-bearing mice were treated with intratumoral injections of either PBS, trypsin, or collagenase/dispase 1 day before a single intratumoral injection of 50 μl of saline containing 1×10^9 pfu Ad-HSV-tk (Fig. 2). All animals received GCV (50 mg/kg) i.p., twice daily for 10 days, starting 1 day after the administration of recombinant adenovirus. We found that trypsin or collagenase/dispase pretreatment enhanced the response of tumors to treatment (Fig. 2). Over the entire 50-day observation period, similar effects were found with the treatment of xenografts from both the U87 and U251 cell lines. To document further this therapeutic enhancement, we sacrificed animals 50 days after the initiation of the tumor xenografts and weighed the s.c. tumors. There was a significant decrease in the weight of U87 and U251 xenografts treated with either trypsin or collagenase/dispase
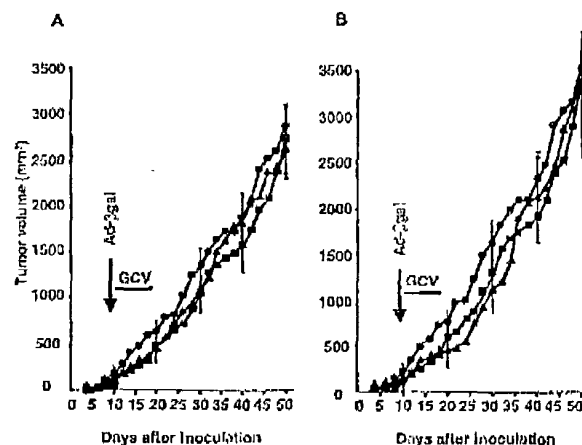

Fig. 1. Effect of protease pretreatment on xenograft growth. Female BALB/c homozygous nude mice, 6 weeks of age, were s.c. inoculated with cells from the human GBM-derived cell line, U87 (7×10^6 cells). On day 9 after the inoculation of cells, animals with xenografts measuring 0.6–0.7 cm in diameter were pretreated with either intratumoral injections of 100 μl of PBS (●), PBS containing trypsin (100 μg; ■), or PBS containing a mixture of collagenase/dispase (10 μg; ▲). Ad-βgal (1×10^9 pfu in 50 μl of saline) was administered 1 day after protease pretreatment. Animals inoculated with adenovirus were further treated with GCV for 10 days starting 24 h after virus inoculation. GCV was administered by i.p. injection twice daily, at a dose of 50 mg/kg injection. U87 (A) or U251 (B) s.c. flank xenografts in immunodeficient mice were measured using calipers until day 50, and the tumor volume was calculated. Each point represents the average volume from seven mice. Data are shown as the means; bars, SD.
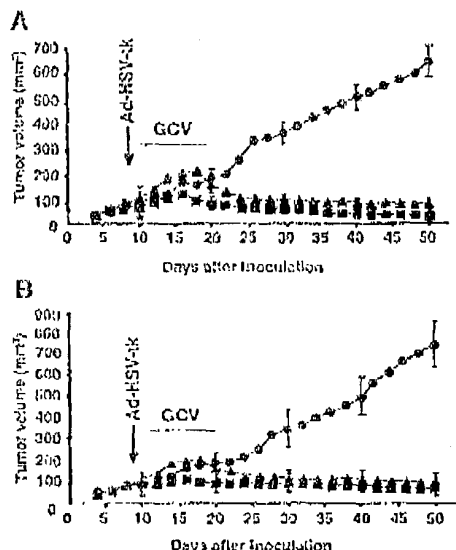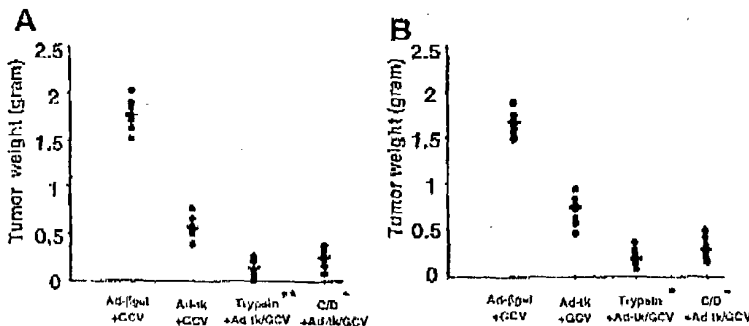
PROTEASE-ENHANCED GENE THERAPY

Fig 2 Effect of protease pretreatment on Ad-HSV-tk/GCV treatment. After the establishment of U87 (A) or U251 (B) xenografts, mice were pretreated with intratumoral injections of 100 µl of either PBS (●), 10 µg trypsin (■), or 100 µg collagenase/dispase (▲). One day after protease treatment, all mice were inoculated intratumorally with Ad-HSV-tk (50 µl of 1 × 10⁹ pfu) and treated with GCV for 10 days. All mice received GCV twice daily at a dose of 50 mg/kg. The tumor growth kinetics were determined by measuring daily the volume of U87 and U251 flank xenografts up to day 50 after cell inoculation. Each point represents the average volume from seven mice. Data are shown as the means. bars SD

injection prior to the initiation of gene therapy compared with tumors found in animals treated with Ad-HSV-tk/GCV alone (Fig. 3). A similar decrease in the weight of these xenografts was observed with collagenase/dispase pretreatment.

We analyzed the survival of mice receiving trypsin pretreatment combined with Ad-HSV-tk/GCV. There were 15 animals in each treatment group. Animals in all treatment groups were sacrificed when the xenografts reach 20 mm in their largest diameter and were scored as having died (Fig. 4). As a result of Ad-HSV-tk/GCV therapy, survival was prolonged compared with a mock-treated control group. Mice receiving trypsin pretreatment combined with Ad-HSV-tk therapy showed an improved survival when compared with Ad-HSV-tk/GCV alone. The analysis between mice receiving trypsin pretreatment with Ad-HSV-tk/GCV and Ad-HSV-tk/GCV alone was statistically significant and indicated that trypsin pretreatment contributed to the therapeutic effect of Ad-HSV-tk/GCV alone ($P = 0.03$).

Effect of Trypsin on Rat and Human Brain Tissue. To evaluate the effect of protease pretreatment on the architecture of normal brain tissue, rat brain specimens were evaluated histologically 24 h after inoculation with saline or trypsin. Pretreatment of these normal tissues with proteases resulted in no apparent morphological changes (Fig. 5). We also evaluated the effect of protease on normal human brain tissue (kindly provided by Dr. Andy Bollen, Department of Pathology, University of California San Francisco, San Francisco, CA). After the inoculation of trypsin into human brain autopsy material and incubation at 37°C for 4 h, we were unable to detect any histological evidence of protease-mediated tissue damage in either white or gray matter (data not shown).

Effect of Protease Pretreatment on the Metastatic Potential of GBM Xenografts. Our experiments indicated that the administration of intratumoral protease enhanced the efficacy of adenovirus-mediated gene therapy. We believe that this enhancement resulted from protease-mediated degradation of extracellular matrix proteins found in the tumor tissue. Although the extracellular matrix of brain is distinctly different from that of brain tumors, we examined mice after treatment for evidence of increased tumor invasiveness and enhanced metastatic potential in three ways: H&E staining of the lymph nodes; fluorescence microscopy of tumors to identify GFP-transfected tumor cells; and PCR analysis of mouse tissues for evidence of human
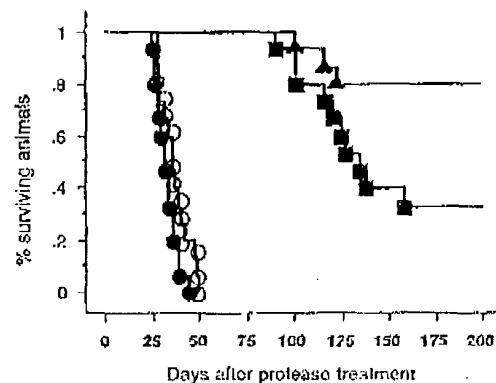

Fig. 4. Effect of trypsin pretreatment and Ad-HSV-tk/GCV treatment on survival of mice bearing U87 xenografts. BAL B/c/nude mice bearing U87 xenografts 0.6–0.7 cm in diameter were pretreated with either intratumoral injections of PBS or PBS containing bovine trypsin (100 µg). Twenty-four h later, 1 × 10⁹ pfu of Ad-HSV-tk or PBS were injected into the xenografts. On the next day, GCV treatment (50 mg/kg, twice daily) was started and was continued for 10 days. ●, PBS + PBS/GCV; ○, trypsin + PBS/GCV; ■, PBS + Ad-HSV-tk/GCV; ▲, trypsin + Ad-HSV-tk/GCV. Animals were sacrificed when xenografts were 20 mm in diameter. Tumor-free survival was designated as the time after treatment initiation when the xenograft was <20 mm. Each group includes 15 mice.

Fig 3 Effect of protease pretreatment on Ad-HSV-tk/GCV treatment. After the establishment of U87 (A) or U251 (B) xenografts, mice were pretreated with intratumoral injections of 100 µl of either PBS (●), 10 µg trypsin (■), or 100 µg collagenase/dispase (▲). One day after protease treatment, all mice were inoculated intratumorally with Ad-HSV-tk (50 µl of 1 × 10⁹ pfu) and treated with GCV for 10 days. Mice were pretreated with either intratumoral injections of 100 µl of PBS, 10 µg trypsin, or 100 µg collagenase/dispase (C/D). One day after protease treatment, all mice were inoculated intratumorally with Ad-HSV-tk (50 µl of 1 × 10⁹ pfu) and treated with GCV for 10 days. Tumors from U87 and U251 xenografts were resected and weighed 50 days after the xenografts were initiated. Each point represents the weight of an individual tumor. Horizontal bash mark median weight for each treatment group. All treatment groups were compared of seven mice. *, P < 0.05, **, P < 0.01 (unpaired t test, compared with Ad-HSV-tk/GCV treated alone)

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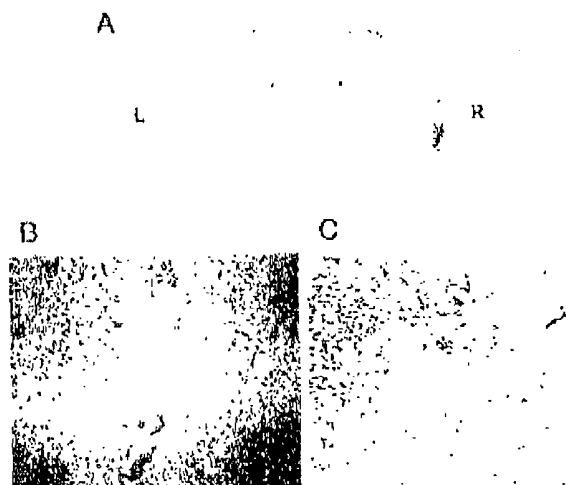
A

L   R

B   C

Fig. 5 Evaluation of protease effect on normal rat brain tissue architecture. *A*, rat brain specimens were evaluated histologically by H&E staining 24 h after the inoculation of saline (10 µg, *L*, left) or trypsin (100 µg; *R*, right) into the basal ganglia. *B* and *C*, high magnification (×200) of normal rat brain specimens after inoculation of saline (*B*) and trypsin (*C*). In each panel, the needle track was observed in the central area with a small hemorrhage.

xenograft DNA. We evaluated abdominal lymph nodes from euthanized mice bearing GFP-transfected U251 xenografts in three treatment groups: mock-treated, Ad-HSV-tk/GCV alone; and Ad-HSV-tk/GCV combined with trypsin pretreatment. Five-µm histological sections of lymph nodes from four mice in each group were fixed in 10% formalin and stained with H&E and examined. We detected no evidence of metastasis by histological examination of these lymph nodes (data not shown).

To look for evidence of metastatic spread of tumor after the treatment of mice bearing GFP-transfected U251 xenografts, we sought evidence of GFP in major organs by fluorescence microscopy. We found no GFP expression in the major organs of these three treatment groups (data not shown). We extracted DNAs from the lung, liver, and brain of four tumor-bearing animals. We then performed PCR analysis of mouse genomic DNA from each of these tissues from four mice in each treatment group seeking evidence of GFP DNA. As shown in Fig. 6, we did not observe amplification of DNA with GFP primers from any organ of a mouse bearing GFP-transfected xe-

nografts. Thus, there was no sign of metastasis either by histological inspection of lymph nodes, microscopic evaluation of major organs, or PCR evaluation of multiple major organs. This suggests that there was not increased tumor invasiveness or enhanced tumor cell spread after protease pretreatment of these GBM-derived xenografts.
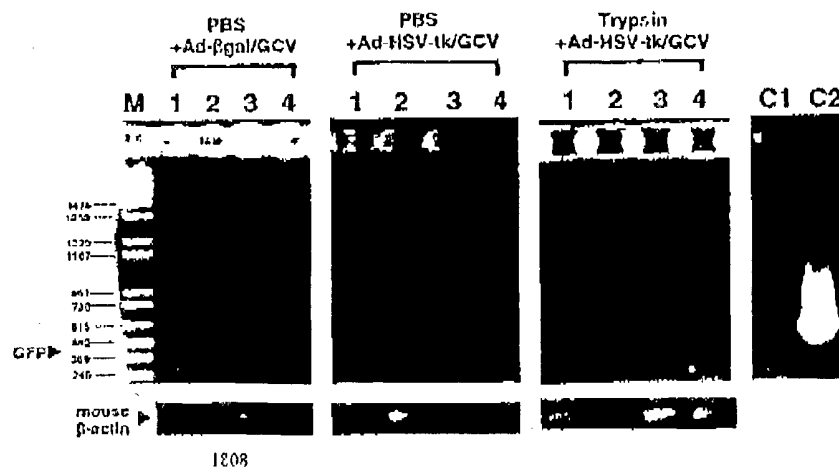
Discussion

A major drawback to current virus-mediated gene therapies is low *in vivo* gene transduction efficiency. Because all gene therapy strategies are compromised by this problem, new strategies are needed to improve viral vector infection efficiency. We have evaluated the potential of protease pretreatment to enhance the efficacy of a gene therapy strategy that relies heavily on the extent of virus-mediated gene transfer. Previously, we evaluated the effect of administering a low dose of trypsin (100 µg) or collagenase/dispase (10 µg) 1 day prior to adenovirus inoculation on virus-mediated gene transfer in human tumor xenografts. Both trypsin and collagenase/dispase enhanced the transduction of recombinant adenovirus (18). To determine whether such an enhancement could contribute to therapeutic outcome, we evaluated the antitumor activity of these pretreatments in combination with Ad-HSV-tk/GCV therapy.

Previous *in vivo* studies have demonstrated the inhibition of GBM tumor growth in animal models after infection with viral vectors carrying the HSV-tk gene and GCV administration (22-24). As shown in Figs. 2 and 3, Ad-HSV-tk/GCV coupled with protease pretreatment resulted in a significantly enhanced antitumor activity against U87 and U251 GBM xenografts when compared with Ad-HSV-tk/GCV gene therapy alone. Treatment with either trypsin (100 µg) or collagenase/dispase (10 µg) was useful in enhancing the effectiveness of the viral vector-mediated Ad-HSV-tk/GCV gene therapy (Figs. 2–4).

A potential explanation for the increased virus infection after pretreatment of tumors with protease is that modifications in the extracellular matrix occur as a result of enzyme-mediated protein degradation. Protease that enhances the therapeutic effect of Ad-HSV-tk/GCV by destruction of the brain tumor extracellular matrix will only be advantageous if the protease does not directly damage adjacent normal tissue. To determine whether the architecture of normal brain was changed by protease pretreatment, we applied trypsin directly to normal rat and human brain tissue and examined them microscopically. There was no observable effect of protease administration on the morphology of these tissues. This suggests that protease might modify brain tumor extracellular matrix while leaving normal brain tissue intact.

Fig. 6. Evaluation of mice bearing GFP-transfected U251 xenografts for metastatic tumor spread. Ad-HSV (5 to 50 µl saline (1 × 10⁹ pfu) was administered intracranially into nude mice bearing GFP-transfected U251 brain xenografts. Fifty days later, we collected major organs and xenografts from these animals, prepared DNA, and examined each tissue by PCR using primers specific for the transfected GFP gene. PCR reactions were evaluated by electrophoresis in 1.5% agarose gels, and the 484-bp (U251) and 571-bp (mouse β-actin) PCR products were viewed by ethidium bromide staining. PCR analysis of each tissue was performed from four mice in each treatment group. Each panel shows representative results from one mouse in each group. Plasmid DNA containing GFP was used in the positive control, whereas the negative control contained no template. In each panel *Lane 1*, GFP-transfected U251 mice xenograft; *Lane 2*, mouse lung; *Lane 3*, mouse liver; *Lane 4*, mouse brain; *Lane C1* negative control from U251 cells; *Lane C2*, positive control (plasmid DNA containing GFP); *Lane M*, DNA marker fragments of the size indicated by bp number.

PBS +Ad-βgal/GCV   PBS +Ad-HSV-tk/GCV   Trypsin +Ad-HSV-tk/GCV

M 1 2 3 4   1 2 3 4   1 2 3 4   C1 C2

GFP▶

mouse β-actin▶

1808

Extracranial metastases arising from primary brain tumors are very unusual. However, there have been reports of GBM that has spread to mediastinal lymph nodes, lung parenchyma, liver, and brain (25-29). The infrequency of such findings is thought to reflect the unlikely invasion of GBM-derived cells to tissues outside the neuroaxis. Because protease has the capacity to disrupt extracellular matrix, a potential side effect of using proteases prior to virus inoculation is the invasion of tumor cells into adjacent areas and the initiation of metastatic tumor spread. We performed both a histological evaluation and a PCR-based examination of lymph nodes and major organs of xenograft-bearing animals to assess metastatic tumor spread after protease pretreatment. We could not detect any evidence of an increased potential for metastatic tumor spread. These results suggest that our strategy of protease pretreatment could be safely used in conjunction with current virus-mediated gene therapy.

Our findings support the use of protease pretreatment as a strategy for enhancing the effectiveness of gene therapy for brain tumors without adversely affecting normal brain tissue. Our results demonstrate the therapeutic potential of protease pretreatment coupled with Ad HSV-tk/GCV gene therapy in a brain tumor model. The potential of this strategy to enhance the therapeutic outcome of virus-mediated gene therapy provides a foundation for future clinical trials and may lead to improved treatment of human brain tumors. Because the effectiveness of all gene therapeutic strategies rely heavily on increased gene transduction efficiency, it will be important to determine whether our findings can be extended to a variety of current virus-based vectors. Further study to clarify the mechanism by which protease pretreatment enhances adenovirus-mediated gene therapy and its evaluation of its application for other virus-mediated gene therapy approaches to the treatment of malignant tumors is needed to develop further this new experimental strategy.

Acknowledgments

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